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Structure and Catalytic Activity of Crystalline Yeast Phosphoglyceric Acid Mutase

RYUZO SASAKI

1966

Structure and Catalytic Activity of Crystalline Yeast Phosphoglyceric Acid Mutase

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1966

CONTENTS

ABBREVIATIONS

PGA mutase	Phosphoglyceric acid mutase
D-2,3-PGA	D-2,3-diphosphoglyceric acid
D-2-PGA	D-2-phosphoglyceric acid
DL-2-PGA	D-2-phosphoglyceric acid
D-3-PGA	D-3-phosphoglyceric acid
DFP	Diisopropylfluorophosphate
TCA	Trichloroacetic acid
p-CMB	Para-chloromercuribenzoate
TNBS	Trinitrobenzenesulfonate
EDTA	Ethylenediamine-tetracetic acid
Tris	Tris(hydroxymethyl)aminomethane
Cpase A	Carboxypeptidase A
$(\alpha)_D$	Specific rotation at 589 mμ
$(\alpha)_\lambda$	Specific rotation at wavelength λ
λ	Wavelength (mμ)
$\overline{M}$	Mean residue weight
$[m']$	Mean residue rotation
ε	Molar extinction coefficient
S	Svedberg unit ($1S = 10^{-13}$ sec)
$s_{20,\,w}$	Sedimentation coefficient in water at 20°

CHAPTER I

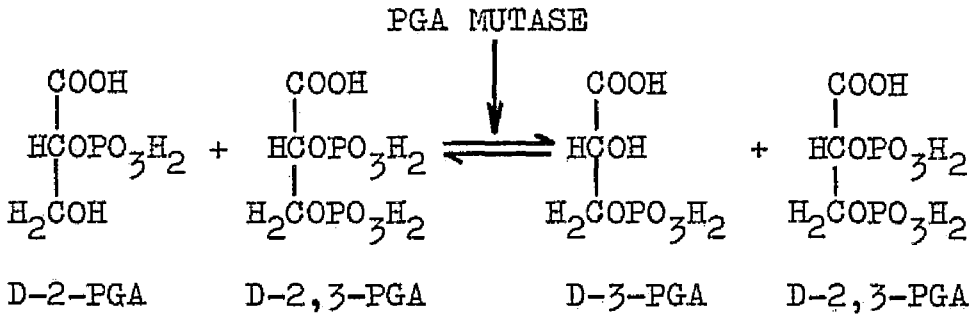
INTRODUCTION

It is widely accepted that catalytic power of enzyme proteins correlates closely with the structure of these large molecules. Considerable progress has been made in the development of a quantitative treatment of protein structure. For such studies it is neccessary to have pure enzymes in a considerable large amount.

In the present study, crystalline phosphoglyceric acid mutase (E.C. 2.7.5.3. D-2,3-diphosphoglycerate : D-2-phosphoglycerate phosphotransferase) from Saccharomyces cerevisiae (baker's yeast) was chosen for the study of the relationship between catalytic power and conformation of enzyme proteins. This enzyme catalyzes an interesting reaction and the crystalline preparation obtained from autolysates of baker's yeast provides very interesting problems in the field of protein chemistry as described below.

Phosphoglyceric acid mutase (PGA mutase)

catalyzes the reversible transfer of a phosphate group between the 3- and 2- positions of D-glyceric acid (1,2). In the case of PGA mutase from yeast and muscle, the reaction must be started by the addition of D-2,3-diphospho-glyceric acid (D-2,3-PGA)(3,4). The diphosphate remains constant during the reaction and acts catalytically as a coenzyme of the reaction. The process is a transfer in which coenzyme is converted into product and substrate into coenzyme, a very interesting type of reaction. PGA mutase from wheat and rice girm, however, does not need a cofactor (5,6).



The yeast enzyme has been crystallized by Rodwell et al (7) and, by a modification of the procedure, by Chiba and Sugimoto (8). Both preparations contained five components which are distinguishable electrophoretically, although

behaved as one component in the ultracentrifuge (9,10). All components are PGA mutase proteins and have the same enzymic properties except specific activity (11,12). Specific activities of the components range from 5,300 to 200 in order of increasing negative ionic mobility. It has been evidenced that electrophoretic heterogeneity is caused by enzymic modification of PGA mutase during autolysis of baker's yeast (13).

In the present paper, the mechanism of the enzymic modification is studied in detail. Moreover, the correlation between the protein structure of PGA mutase and its catalytic activity is investigated by using several usefull techniques.

CHAPTER II

EXPERIMENTAL

Determination of Phosphoglyceric Acid Mutase Activity

DL-2-phosphoglyceric acid (DL-2-PGA) was synthesized by oxidation of sodium β-glycerophosphate according to the method of Kiessling (14) and purified as described by Warburg and Christian (15). D-2,3-PGA was isolated from pig erythrocyte according to the procedure of Greenwald (16).

Enzyme activity was determined by the polarimetric method (8). This method is based on the difference in optical rotation of D-2-PGA and D-3-phosphoglyceric acid (D-3-PGA) in a molybdate solution. Since $(\alpha)_D$ of D-3-PGA in the solution containing molybdate ion is -745° and that of D-2-PGA is -68°, the relation showed in Table I is obtained.

The assay system contained 165 μmoles of DL-2-PGA, 0.5 μmole of D-2,3-PGA, 500 μmoles of sodium acetate buffer (pH 5.9) and enzyme in a final volume of 5 ml. After incubation at 25°

for 10 minutes, the reaction was stopped by the addition of 5 ml of 20 % ammonium molybdate.

Table I. Basal Relation in Enzyme Assay

Substrate	α
DL-2-PGA	-677 X

α represented optical rotation of the mixture of 2- and 3-acids. X represented the fraction of the substrate converted (per cent).

Optical rotation was measured with a photoelectric polarimeter attached to a Beckman DU spectrophotometer using a 1-dm tube and the amount of D-3-PGA formed was calculated according to the relation in Table I. Under these conditions, one enzyme unit was defined as that amount which catalyzed the formation of 1 μmole of D-3-PGA from D-2-PGA. Specific activity was expressed in unit per mg of protein. The protein content in the assay solution was determined spectrophotometrically. The extinction coefficients, $E^{1\%}_{1\,cm}$ at 280 mμ of component I and V, were determined to be 14.2 and 14.9 respectively by measurements of dry weight.

Preparation of Crystalline Enzyme

Crystalline enzyme preparations obtained from autolysate of baker's yeast contain five components (I~V) which are electrophoretically distinguishable and which exhibit different specific activities (10). Each component is a PGA mutase (12). Component I (the highest specific activity and lowest electrophoretic mobility) is the native PGA mutase in yeast cells and is modified into other components by PGA mutase-modifying enzyme (13). Individual components have been seperated preparatively by vertical zone electrophoresis, and crystallized (11).

Native PGA mutase was also obtained directly from yeast by low-temperature extraction from an acetone powder. The dried powder was suspended in 4 volumes of 0.05 M sodium phosphate buffer, pH 7.0 and after standing at 2° for 15 hours, the supernatant liquid was treated as described by Chiba and Sugimoto (8). The crystalline PGA mutase obtained consisted almost entirely component I, but the yield was low.

It was found that DEAE-cellulose chromatography was suitable for the preparation of component I (native PGA mutase). The unfractionated preparation (containing five components) was dialysed against 0.002 M sodium phosphate buffer, pH 7.0 and applied to a DEAE-cellulose column (2 X 30 cm) which had been equilibrated with the same buffer. Chromatography was performed at 5° with a linear gradient of ammonium sulfate in the same buffer. A flow rate was 50 ml per hour. Component I was eluted at 0.005-0.007 M ammonium sulfate. The modified PGA mutases (component II, III and IV) were eluted in that order ; component V finaly eluted at 0.016-0.02 M ammonium sulfate.

In the present work, native PGA mutase (component I, specific activity : 5,300) was seperated chromatographically from unfractionated crystalline enzyme prepared from a 3-hour autolysate. The crystalline PGA mutase preparation containing five components (specific activity : 3,600) obtained from a 6-hour autolysate was used as the unfractinated

crystalline PGA mutase. A limit PGA mutase preparation (component V, specific activity : 200) was prepared in high yield from a 24-hour autolysate. Autolysis of baker's yeast and purification and crystallinzation of enzyme were carried out by the method reported (8).

The crystalline enzyme was usually kept as a suspension in ammonium sulfate mother liquor at 0°. Under these conditions, activity unchanged for a few month.

CHAPTER III

ENZYMIC MODIFICATION

1. Introduction

Crystalline PGA mutase is an ultracentrifugally homogeneous protein, but contains five components with different electrophoretic mobilities (9,10). Each component has the same crystalline form, and is a PGA mutase. The specific activities of the components range from 5,300 (component I) to 200 (component V) in order of increasing negative electrophoretic mobility. Several properties (pH optima, equilibrium constants and Michaelis constants for D-2,3-PGA) of the individual components are almost identical (12). Short autolysis times favored a greater proportion of components with lower electrophoretic mobilities and higher specific activities (13). Crystalline preparation obtained by low-temperature extraction from an acetone powder of yeast consisted almost of component I as described in Chapter II.

From these results, it is concluded that component I is the native PGA mutase in yeast cells and is modified into the other components during autolysis. An unknown enzyme which modifies the PGA mutase was found to be present in yeast extract. This enzyme was called PGA mutase-modifying enzyme (13).

In this Chapter, the properties and action of the modifying enzyme are studied to shed light on the mechanism of the enzymic modification of PGA mutase. Properties of component I and V are presented. Moreover, actions of highly purified peptide hydrolases on PGA mutase are also studied.

2. Materials and Methods

Determination of Phosphoglyceric Acid Mutase-Modifying Enzyme Activity

The reaction mixture, in a final volume of 1 ml, contained 10,000 units of unfractionated crystalline PGA mutase (specific activity : 3,600), 50 μmoles of sodium phosphate buffer, pH 7.0 and the modifying enzyme diluted adequately. The reaction was carried out at 38°

and started by the addition of the modifying enzyme. Cold 0.02 M ammonium sulfate solution was added to the reaction mixture. The dilution and cooling stopped the reaction, and residual PGA mutase activity in the reaction mixture was determined by the method as described in Chapter II. The control contained all reagents except the modifying enzyme. Zero-order kinetics were observed under these conditions untill 40 % of the PGA mutase activity were lost. One unit of the modifying enzyme was defined as that amount of the enzyme that gave 1 unit loss of PGA mutase activity per minute under the above conditions.

Preparation of Phosphoglyceric Acid Mutase-Modifying Enzyme

An extract of baker's yeast was obtained from a 3-hour autolysate. The modifying enzyme in the extract was activated in the refrigerator for 1 month as described later. To 1 liter of the solution 570g of solid ammonium sulfate were added with stirring. The precipitate was collected by centrifugation (10,000 X g, 60 min.),

and dissolved in water. The solution was passed through a column of Sephadex G-50 equilibrated with 0.005 M sodium phosphate buffer, pH 7.0. The resultant preparation contained 6,900 units of the modifying enzyme activity per mg of protein. Protein was determined by the method of Warburg and Christian (15).

3. Results

a. Action of Phosphoglyceric Acid Mutase-Modifying Enzyme

Unfractinated crystalline PGA mutase, 200 mg was incubated at 38° with 30 ml of yeast extract obtained from a 3-hour autolysate. After five hours, 5.5 % of the original activity were remained. The modified PGA mutase in the incubation mixture was purified and crystallized by the procedure reported (8). Electrophoretic patterns of the original and modified PGA mutases are shown in Fig. 1. The PGA mutase protein, composed of five components (I, II, III, IV, and V), was converted to component V by the PGA mutase-modifying enzyme. Moreover, the limit product was confirmed to be component V. Yeast extract treated with 10^{-3}M diisopropylfluorophosphate (DFP) for 60 minutes at 25° did not bring about this change and activity remained unchanged.

A difference of electrophoretic mobility may be caused by alteration of the molecular weight, conformation or net charge of a protein. The individual PGA mutase components, however, have the same crystalline form (11) and show

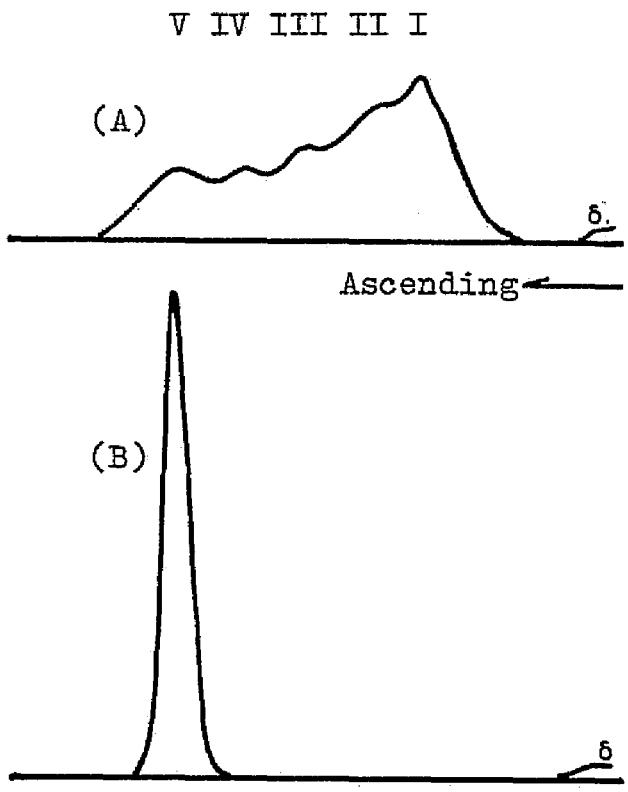


Fig. 1. Moving Boundary Electrophoretic Patterns of Unfractionated and Limit-modified PGA Mutase.

(A), Unfractionated PGA mutase. (B), Limit-modified PGA mutase. Electrophoresis was carried out in a barbital buffer at pH 7.0 and 0.1 ionic strength with a potential gradient of 4.5 volts per cm for 14,400 seconds. The patterns were obtained from a 2 % protein solution in (A) and a 1.4 % solution in (B) at 0.7°.

identical sedimentation properties as described later. Therefore, it is presumed that the differences in electrophoretic mobility are related to the number of ionic groups. The differences in charge between adjacent components (e.g component I and II, II and III etc.) were calculated to be equivalent to one or two groups (17). The following four mechanisms for the action of PGA mutase-modifying enzyme must be considered :

	Component I (native enzyme)		Component II—V (more negatively charged protein)
(a)	Protein-(n phosphate) + m phosphate	→	Protein-[(n+m)phosphate]
(b)	Protein-COO-sugar + H_2O	→	Protein-COO^- + Sugar + H^+
	or Protein-NH_3^+ + Sugar	→	Protein-NH-sugar + H^+ + H_2O
(c)	Protein-$CONH_2$ + H_2O	→	Protein-COO^- + NH_4^+
(d)	Protein	→ (limited proteolysis)	Modified Protein + Amino acid including, or peptides containing, basic amino acids

In the case of (a), PGA mutase is a phosphoprotein. Component I has the lowest content of phosphate groups and the modified PGA mutases have more content in that order. Modifying enzyme is an enzyme which catalyzes the reaction of phosphorylation. In the case of (b), PGA mutase is a sugarprotein. Modifying enzyme catalyzes hydrolysis of ester-likage between carboxyl groups in PGA mutase and sugars or the condensing reaction of amino groups and sugars. The appearance of the modified PGA mutases is attributable to the release of ionizable carboxyl groups or the decrease of ionizable amino groups. In the case of (c), modifying enzyme is a proteinamidase. The modified PGA mutases are derived from component I by the increase of ionizable carboxyl groups due to hydrolysis of amide groups. In the case of (d), modifying enzyme is a kind of peptide hydrolase. The differences of each component in electrophoretic mobility are caused by the different content in basic amino acids.

The phosphate contents of crystalline preparations (component I and V, and unfractionated

crystalline PGA mutase) were determined. The phosphate contents of these preparations were 0.1 or less equivalents of phosphate per mole of enzyme. Moreover, the Tillman and Bial reactions revealed that PGA mutase had no bound sugars. From these results and amino acid analyses as described later, it is concluded that PGA mutase is not a conjugated protein. Therefore, possibilities of (a) and (b) which were proposed for the reaction mechanism of modifying enzyme could be excluded.

b. Quantitative Identification of Product Released by Phosphoglycric Acid Mutase-Modifying Enzyme

The PGA mutase-modifying enzyme is either a protein-amidase or a proteolytic enzyme ; if a protein-amidase, liberation of ammonia should occur during the modification reaction. Table I shows that the PGA mutase-modifying enzyme is not a protein-amidase and that the differences in the electrophoretic mobilities are not caused by changes of amide content. Limited proteolysis is the only likely process catalyzed by the modifying enzyme.

Table I

Liberation of Ammonia by Modification[a]

Incubation time (min.)	PGA mutase activity (%)	NH_3 found	NH_3 liberated
		Mole per mole of PGA mutase	
0	100	0.16	
80	5.5	0.65	0.49

[a] One ml of the incubation mixture contained ; 10 mg of native PGA mutase eluted from a Sephadex G-50 column ; 150 µg of the modifying enzyme ; 20 µmoles of sodium phosphate, pH 7.0. The reaction was carried out at 38°. Ammonia was determined directly by the indophenol method (18).

In fact, it was found that amino acids and peptides are released during the modification of PGA mutase in analysis of the 5 % trichloroacetic acid (TCA)-soluble fraction of the incubation mixture with ninhydrin (19). Figure two shows the release of amino acids and peptides during the modification of the native PGA mutase. When the modifying enzyme was pretreated with DFP, amino acids and peptides were not formed and mutase activity was not lost. Amino acids and peptides released after the native PGA mutase was completely converted to component V were analysed with an automatic amino acid analyzer.

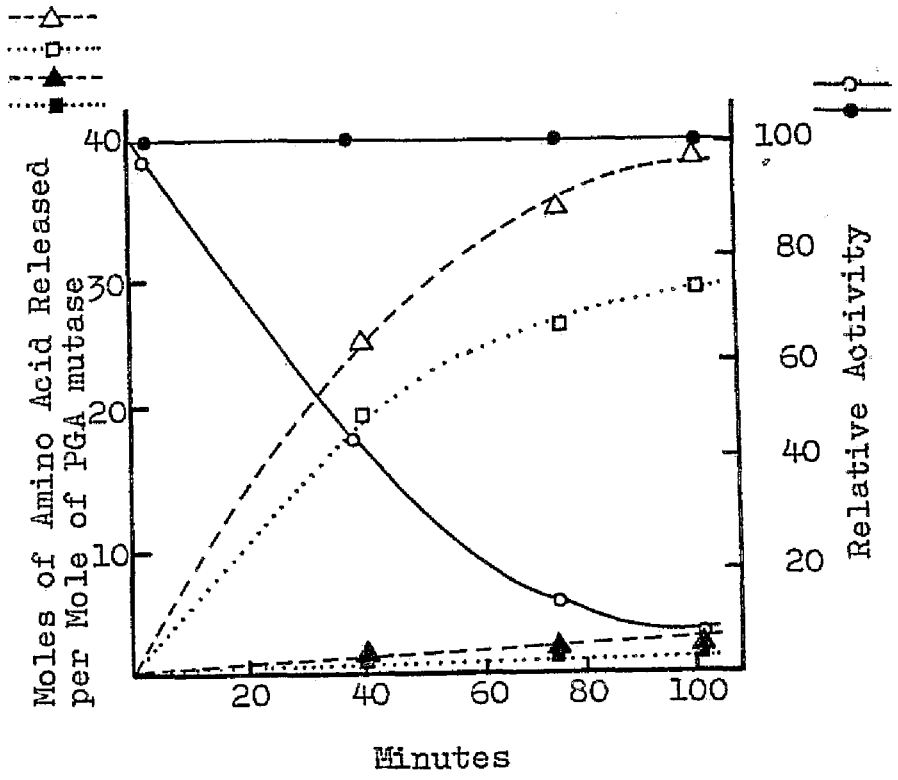


Fig. 2. Relation between PGA Mutase Activity and Amino Acid and Peptide Release during Modification

(o □ △) : Modifying enzyme without treatment was used. (—o—) PGA mutase activity. (···□···) Amino acid and peptide, not hydrolyzed. (-△-) Amino acid, after hydrolysis in 6 N HCl for 24 hours at 105°. (● ■ ▲) : Modifying enzyme treated with DFP was used. (—●—) PGA mutase activity. (···■···) Amino acid and peptide, not hydrolyzed. (-▲-) Amino acid, after hydrolysis in 6 N HCl for 24 hours at 105°. Prior to the reaction, the native PGA mutase was subjected to gel filtration on a Sephadex G-50 column. One ml of the reaction mixture contained : 2.9 mg of native PGA mutase ; 35 µg of modifying enzyme ; 20 µmoles of sodium phosphate, pH 7.0. The reaction was carried out at 38°. The reaction was stopped by adding 1 ml of 10 % TCA and TCA in the supernatant was removed by ether extraction. Amino acid and peptide in the supernatant were determined as leucine equivalent by the ninhydrin method (19).

Table III

Quantitative Identification of Amino Acid Released during Modification[a]

Amino acid	Mole of amino acid released per mole of native PGA mutase (112,000)	
	Not hydrolyzed	Hydrolyzed[b]
Aspartic acid	0	4.3
Threonine	0	0.3
Serine		0.5
Asparagine	5.4	—
Glutamine		—
Glutamic acid	0.1	3.7
Proline	0	0
Glycine	4.8	5.6
Alanine	9.5	9.7
Half-cystine	0	0
Valine	3.6	4.1
Methionine	0	0
Isoleucine	0.1	0.1
Leucine	0.2	0.4
Tyrosine	0	0
Phenylalanine	0	0
Tryptophan	0	—
Lysine	6.3	7.4
Histidine	0	0
Ammonia	0.4	7.1
Arginine	0	0

[a] Nine ml of the incubation mixture contained : 25.6 mg of native PGA mutase eluted from a Sephadex G-50 column ; 315 µg of modifying enzyme ; 180 µmoles of sodium phosphate, pH 7.0. The reaction was carried out at 38° for 100 minutes. Under these conditions, native PGA mutase was converted into component V. The reaction was stopped by the addition of 3 ml of 20 % TCA. Amino acid analyses were performed on the TCA free supernatant solution (see legend of Fig. 2).

[b] Hydrolysis of the 5 % TCA-soluble fraction was caried out in 6 N HCl for 24 hours at 105°.

As shown in Table III the composition of a hydrolysate of the 5 % TCA-soluble fraction indicates that the conversion of component I to component V was accompanied by the liberation of about 37 amino acid residues, including 7 lysine residues. This corresponds to 3 to 4 % of the total amino acid residues in the native PGA mutase. The amount of ammonia in the hydrolysate was almost equivalent to the sum of the aspartic and glutamic acid contents. Moreover, in the unhydrolyzed fraction, only small amount of glutamic acid, aspartic acid and ammonia were detected, but glutamine and asparagine were found. These results show that ammonia in the hydrolysate derived from amide and that most of the glutamic and aspartic acid was present in the amide form. The ultraviolet absorption of the 5 % TCA-soluble fraction showed that neither tyrosine nor tryptophan residues were released. Only lysine among the basic amino acids was released. Consequently, the increase in negative electrophoretic mobility during modification was caused by the liberation of lysine.

c. Properties of Native and Modified PGA Mutase

Amino Acid Composition of Component I and V

The amino acid compositions of component I and V were determined. Before analysis, the crystalline enzyme was dissolved in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride. The solution was passed through a column of Sephadex G-50 equilibrated with the same buffer for removal of ammonium sulfate. One ml of sample solution containing 3 to 5 mg of the protein was hydrolyzed for 22 or 70 hours at 105° in sealed glass tube in the presence of argon with 3 ml of 8 N HCl (final concentration, 6 N HCl). This procedure gave a clear and colorless hydrolyzate. The chromatography of the hydrolyzates was performed on a column of Dowex 50 X 8 by essentially the same procedure as described by Moore and Stein (20). A 0.9 X 100 cm column was used for the acidic and neutral amino acid and a 0.9 X 15 cm column for the basic amino acids. Fractions (1 ml) were collected, and the amino acid concentrations in the fractions were

determined spectrophotometrically by the modified ninhydrin method (19). The ninhydrin color produced by proline was read at 440 mμ and that produced by the other amino acids at 570 mμ with a Beckman DU spectrophotometer. The color values for the amino acids given by Moore and Stein were employed in calculating the yields of amino acids (19).

Cysteine and cystine were determined as cysteic acid after performic acid oxidation and hydrolysis of 20 mg of proteins. The determination of SH groups of this enzyme was also performed by the spectrophotometric p-chloromercuribenzoate binding method (21). Measurement of tryptophan was made on an unhydrolyzed protein by the method of Goodwin and Morton (22).

The results are summarized in Table IV. The values given for the hydrolysate obtained after 22 hours and 70 hours represent the average of triplicate determinations. In cases of threonine serine and ammonia, a crrection has been applied by extraporation to zero time of hydrolysis. In cases where an incrase in the amino acid value

Table IV

Amino Acid Composition of Component I and V

Amino acid	Number of residues per mole of PGA mutase		I—V
	Component I (M.W. 112,000)	Component V (M.W. 108,000)	
Aspartic acid	108	103	5
Threonine ------ a	37	36	1
Serine --------- a	61	59	2
Glutamic acid	103	99	4
Proline	65	65	0
Glycine	62	54	8
Alanine	99	81	18
Valine --------- b	67	63	4
Methionine	5	5	0
Isoleucine ----- b	44	43	1
Leucine	112	110	2
Tyrosine	35	35	0
Phenylalanine	23	23	0
Histidine	16	16	0
Lysine	92	85	7
Ammonia (Amide)	70	65	5
Arginine	49	49	0
Half-cystine --- c	1	1	0
Tryptophan --- d	18	18	0

a Extraporated value.

b Value from 70-hours hydrolyzate.

c Dtermined as cysteic acid after performic oxidation and hydrolysis of the enzyme.

d Estimated spectrophotometrically.

was found with longer hydrolysis time, the average values for the 70-hour hydrolyzates have been used (valine and isoleucine).

It is evident that native PGA mutase has a rather high content of proline and a rather low content of sulfer-containing amino acids. One residue of half-cysteine per mole of the enzyme indicates the presence of one residue of cysteine. This result is in agreement with that obtained from the determination of SH groups of the enzyme by the method of Boyer (21). The absence of disulfide bridges is a unique feature of this enzyme, although it has a high molecular weight.

The amino acid composition of component V was found to be similer to that of component I, although lower contents of alanine, lysine, glycine, valine, aspartic acid and glutamic acid were apparent (Table IV). The difference between the amino acid composition of component V and that of component I corresponds closely to the number of the amino acid residues found to be released by the modification. The total molecular weight change on modification was calculated to be about 4,000 (see Table III). Since the molecular weight of component I is 112,000, that of component V is taken as 108,000.

Sedimentation Analyses of Component I and V

The molecular weight of PGA mutase (unfractionated crystals) was reported as 112,000 ($s_{20,w}$ = 6.3) from sedimentation-diffusion studies (9). A similer result was obtained for the molecular weight of component I by application of the Archibald principle (23).

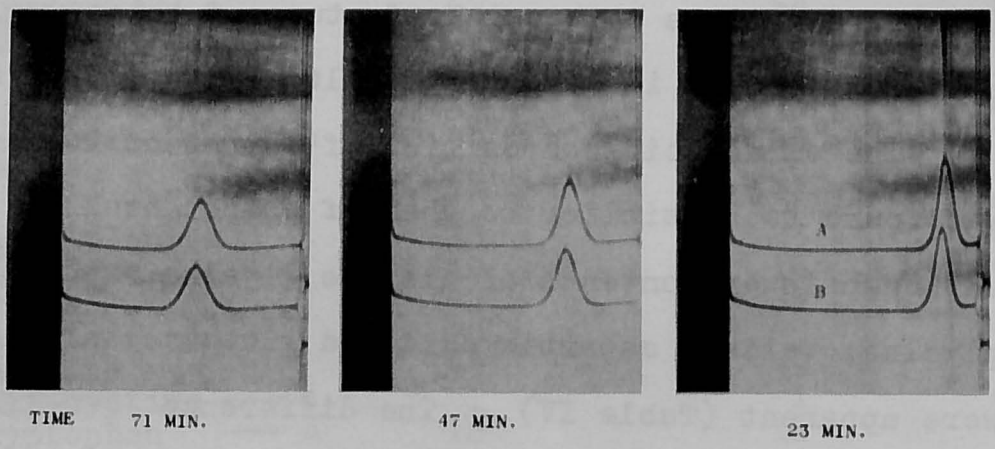


Fig. 3. Sedimentation Velocity Patterns for Native and Limit-Modified PGA Mutase.

(A), native PGA mutase. (B), limit-modified PGA mutase. Both solutions contained 7 mg/ml of the PGA mutase protein in 0.2 M NaCl, 0.04 M sodium phosphate buffer at pH 7.0. The patterns were obtained at 23, 47 and 71 minutes after attaining a speed of 59,780 r.p.m. at an angle of 70° for the shlieren diaphram. Sedimentation was from right to left.

Ultracentrifugal experiments were performed with 0.7 % solutions of native and limit-modified PGA mutase under the same conditions. Both enzymes showed homogeneous shlieren pattern with sharp peaks throughout the duration of a 71 minute run at 59,780 r.p.m. (Fig. 3) and had the same sedimentation coefficients ($s_{20,w}$ at a concentration of 0.7 % : 5.7). As described already, amino acid residues released during the modification correspond to a few percent of total amino acid residues in the native PGA mutase. Without any difference in molecular shape, the native and limit-modified PGA mutase could not be distinguished ultracentrifugally. In other words, the identical ultracentrifugal behaviour of native and limit-modified PGA mutase suggests that extensive change in molecular shape is not caused during the modification.

Optical Rotation during Modification

Measurements of optical rotation of solutions of proteins are very usefull for the investigation of protein structures because this property is

very sensitive to changes in molecular conformation. Evidence for a conformational change during modification was sought by measurements of optical rotation with a photoelectric polarimeter attached to a Beckman DU spectrophotometer. As shown in Fig. 4, little change was observed.

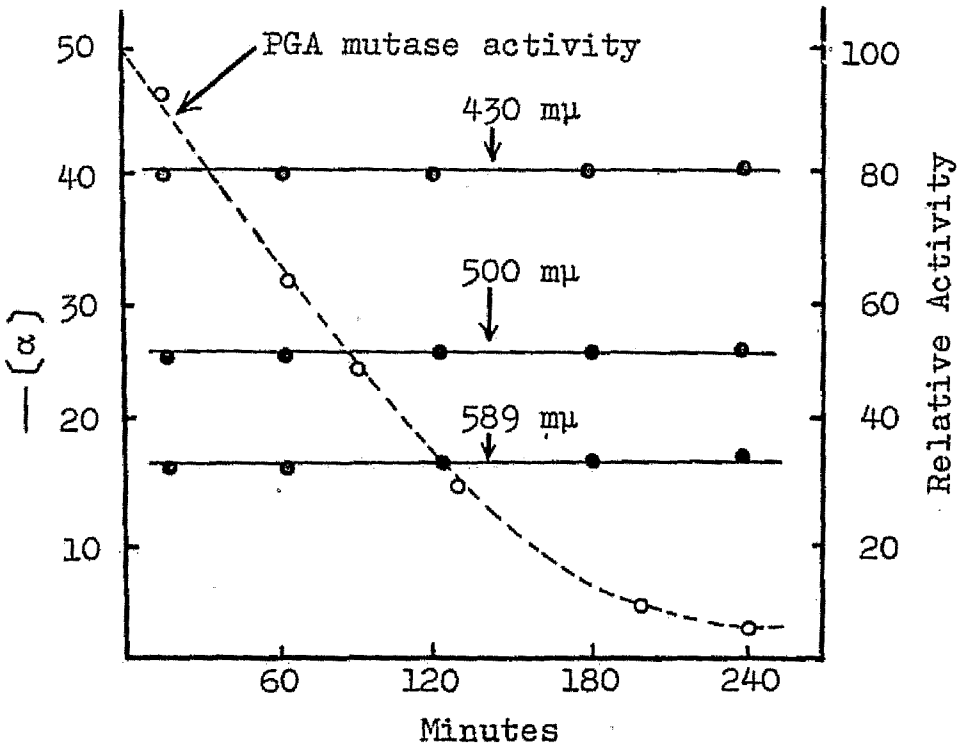


Fig. 4. Optical Rotation of PGA Mutase during Modification.

(---o---) PGA mutase activity. (—•—) Optical rotation. Two ml of the incubation mixture contained : 38.6 mg of native PGA mutase 250 μg of the modifying enzyme ; 40 μmoles of sodium phosphate, pH 7.0. The reaction was carried out at 18°.

Ultraviolet Difference Spectrum during Modification

The ultraviolet absorption spectra of protein solutions in the region 260 to 300 mμ contain contributions from phenylalanyl, tyrosyl and tryptophyl residues. Changes in the environment of these chromophores due to conformational changes of a protein can lead to alterations in the absorption spectrum. Therefore, ultraviolet difference spectrum (260～300 mμ) was measured for the detection of a conformational change during modification. The final concentration of component I was 10^{-5}M in each cuvette (light pass : 1 cm). The modifying enzyme solution was added to one of the cuvettes and the same volume of buffer to another. The incubation was carried out at 20°. Although enzyme activity was lowered to 5 % of the original, there was no change in difference spectrum. Therefore, it is unlikely that the environment around the aromatic chromophores is changed. On the other hand, $E^{1\%}_{1\ cm}$ at 280 mμ of component V was 14.9 from measurements of its dry weight. This value is in reasonable agreement

with a molecular weight for component V of 108,000, compared with 112,000 for component I ($E^{1\%}_{1\ cm}$ at 280 mμ = 14.2), since the products released by the modifying enzyme contain neither tyrosine nor tryptophan.

D-2,3-Diphosphoglyceric Acid Phosphatase Activity

It was found that crystalline PGA mutase obtained from yeast showed D-2,3-PGA phosphatase activity. Since the phosphatase activity was extremely low compared to the mutase activity, there was no difficulty with the assay of PGA mutase. The D-2,3-PGA phosphatase activities of native, unfractionated and limit-modified PGA mutase were determined. The specific activities of the preparations tested were all the same as shown in Table V. Moreover, these preparations possessed the same Michaelis constant for D-2,3-PGA with regard to mutase activity (12). These results reveal that the phosphatase activity is not due to a contaminant in crystalline PGA mutase preparations and that PGA mutase is a double headed enzyme.

It is noteworthy that mutase activity partially destroyed, but D-2,3-PGA phosphatase activity was unchanged by the modification of PGA mutase.

Table V

D-2,3-PGA Phosphatase Activity of PGA Mutase[a]

PGA mutase used	PGA mutase specific activity	µmoles of inorganic phosphate liberated		
		5 min.	20 min.	60 min.
Component I	5,300	0.27	1.07	2.68
Component V	200	0.26	1.09	2.80
Unfractionated PGA mutase	3,600	0.28	1.08	2.64

[a] One ml of the reaction mixture contained : 5 µmoles of D-2,3-PGA ; 100 µmoles of Tris, pH 7.0 ; 1 mg of PGA mutase. The reaction was carried out at 37° and stopped by the addition of 3 ml of 2 N H_2SO_4. Inorganic phosphate in the supernatant was determined by the method of Chen et al (24).

Chemical Modification of Component I and V

It is of interest to determine whether the loss of PGA mutase activity by the action of modifying enzyme is caused by the release of particular amino acid residues. It is noteworthy to pay attention to lysine residues which are released during modification and lead to the appearance of components with different electrophoretic mobilities. With this intention, chemical modification experiments on component I and V were studied by using 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) which reacts with primary amino groups in protein molecule. The amount of TNBS bound to PGA mutase was calculated from absorption at 345 mμ with a molar extinction coefficient of 1.33×10^4 (25).

If lysine residues released during modification are more reactive for TNBS, a difference in the rate of TNBS-binding between component I and component V must be observed. Moreover, if the loss of PGA mutase activity by the action of modifying enzyme is caused by the release of these lysine residues, percent of loss of activity

in component I by TNBS-binding should be larger than in component V. The rate of trinitrophenylation of component I and V is shown in Fig. 5.

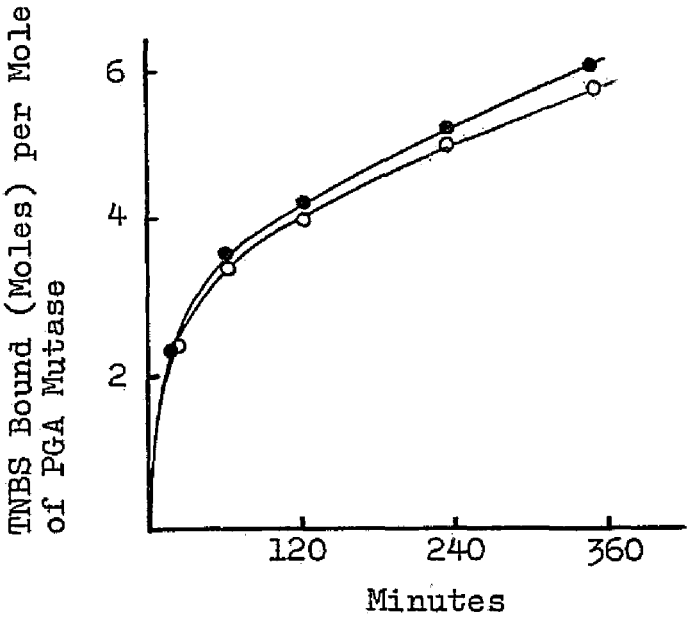


Fig. 5. Time Course of TNBS-Binding with PGA Mutase (Component I and V).

(—●—) Component I. (—○—) Component V. The reaction mixtures contained 5 X 10^{-6}M protein, 5 X 10^{-4}M TNBS and 0.035 M sodium borate-HCl buffer (pH 7.5). The temperature was 0°. Absorption at 345 mμ was measured by using the technique of differential spectrophotometry. Reference cells contained all reagents except PGA mutase. Prior to the reaction, PGA mutase was subjected to gel filtration on a Sephadex G-50 column.

No difference was observed in both components. Both components have three lysine residues reactive for TNBS. Moreover, the effect of TNBS-binding on activity is almost identical as shown in Fig. 6.

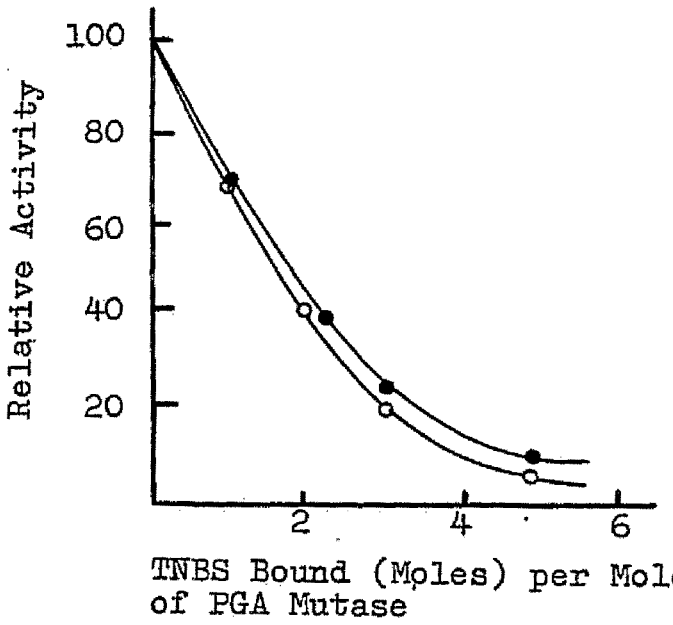


Fig. 6. Effect of TNBS-Binding on PGA Mutase Activity (Component I and V).

(—●—) Component I. (—o—) Component V. The reaction conditions were as in Fig. 5. Aliquots were diluted in cold 0.02 M ammonium sulfate solution at intervals and PGA mutase activity was determined.

These results suggest that both components have three lysine residues which react rapidly with TNBS and that these three lysine residues have a significant role in the action of PGA mutase. However, these reactive lysine residues do not correspond to the lysine released during modification. It is difficult to believe that the differences in specific activity are attributable only to the liberation of lysine residues. The role of reactive lysine residues will be discussed in Chapter IV.

d. Properties of Phosphoglyceric Acid Mutase-Modifying Enzyme

PGA mutase-modifying enzyme activity in an extract obtained from a 3-hour autolysate of yeast increased extensively during 1 month. The extract had casein, carbobenzoxyglycyl-L-phenylalanine and hippuryl-L-lysine hydrolysing activity. The modifying enzyme and carbobenzoxyglycyl-L-phenylalanine and hippuryl-L-lysine hydrolase activitties were completely inhibited by preincubation with DFP, while casein hydrolysing activity partially.

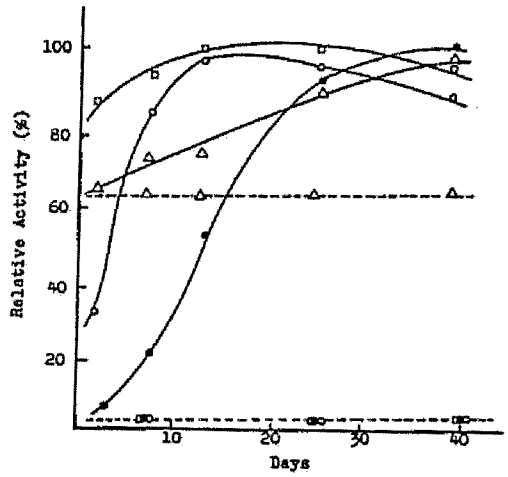


Fig. 7. PGA Mutase-Modifying Enzyme and Peptide Hydrolases Activities in Yeast Extract.

(——) ; Extract without treatment was used. (----) ; Extract treated with 10^{-3} M DFP for 60 minutes at 25° was used. (●) PGA mutase-modifying enzyme activity. (△) Casein-hydrolysing activity. The activity was determined by the method of Kunitz (26). (□) Carbobenzoxyglycyl-L-phenylalanine hydrolysing activity. The activity was determined by the method of Neurath (27). (○) Hippuryl-L-lysine hydrolysing activity. The activity was determined by measurements of liberated lysine according to the method of Work (28).
The extract obtained from a 3-hour autolysate was covered with toluene and stored in the refrigerator at 4°. Before used for activity determinations, the extract was treated by the same method for the modifying enzyme preparation (see page 11). All reactions were carried out in 0.2 M sodium phosphate buffer, pH 7.0. Maximum value of each enzyme activity during storage is expressed as 100 % of activity.

As shown in Fig. 7, the endopeptidase activity whcih was inhibited by DFP and the exopeptidase activities increased in compoany with the activation of the modifying enzyme. On the other hand, the endopeptidase activity which was not inhibited by DFP remained unchanged.

Table VI

Effect of Various Compounds on Modifying Enzyme[a]

Compound added	Final concentration (M)	Inhibition (%)
DFP[b]	1×10^{-3}	100
o-Phenanthroline	2.5×10^{-3}	9
EDTA	1×10^{-2}	0
Casein	10 mg / ml	100
Ovalbumin	10 mg / ml	100
Carbobenzoxy-glycyl-L-phenyl-alanine	2×10^{-2}	100
Hippuryl-L-lysine	5×10^{-2}	100
D-3-PGA	1×10^{-3}	0
DL-2-PGA	1×10^{-3}	0

[a] Compounds were added directly to the modifying enzyme assay mixture unless otherwise indicated.

[b] Preincubation with the modifying enzyme for 60 minutes at 25°.

The pH otimum of the modifying enzyme reaction was 7-8. The activity was not influenced by dialysis against cold distilled water. Table VI shows the effect of various compounds on the modifying enzyme activity. The enzyme was scarcely affected by chelating agents. Various substrates for peptide hydrolases caused strong inhibition. The substrates of the PGA mutase did not protect the enzyme from the action of modifying enzyme.

e. Action of Peptide Hydrolases on Phosphoglyceric Acid Mutase

The effect of highly purified peptide hydrolases on PGA mutase are shown in Table VII. The mutase was not converted into component V, but disappeared with loss activity when exposed chymotrypsin, trypsin and nagarse. The mutase activity and electrophoretic pattern were not affected by DFP-treated carboxypeptidase A (Cpase A). Untreated Cpase A, however, showed a modifying enzyme-like action.

Table VII

Action of Peptide Hydrolases on PGA Mutase[a]

Enzyme added	Concn. of PGA mutase (mg/ml)	PGA mutase / Enzyme (W/W)	Condition	Residual PGA mutase activity (%)	Paper electrophoretic pattern
			Temp., Min.		Component
———	1	—	38°, 120	100	I II III IV V
Modifying enzyme	3.6	60	38°, 60	6	V
Modifying enzyme treated with DFP[b]	3.6	60	38°, 60	100	I II III IV V
Carboxypeptidase A without treatment[c]	3.6	7.5	25°, 120	7	V
Carboxypeptidase A treated with DFP[b,c]	3.6	7.5	25°, 120	100	I II III IV V
Trypsin	1.8	30	38°, 60	0	0
Trypsin	1.8	150	38°, 120	42	Uniformly disappeared
Chymotrypsin	1.8	50	38°, 60	0	0
Chymotrypsin	1.8	240	38°, 120	46	Uniformly disappeared
Nagarse	2.8	50	38°, 5	0	0

[a] All reactions were carried out in 0.02 M sodium phosphate buffer, pH 7.5. Unfractionated crystalline PGA mutase was used as substrate. 0.02 mg of the mutase protein was applied in each paper electrophoresis. Protein was detected with Amide Black 10B [saturated solution in methanol-distilled water-glacial acetic acid (5 : 5 : 1)].

[b] The enzyme was incubated with 10^{-3}M DFP for 60 minutes at 25°.

[c] The carboxypeptidase A (Mann Research Labolatories) solution was prepared by the method of Harris (29).

Table VIII

Quantitative Identification of Amino Acid Released during Modification of Native PGA Mutase by Cpase A[a]

Amino acid	Mole of amino acid released per mole of native PGA mutase(112,000)	
	Not hydrolyzed	Hydrolyzed[b]
Aspartic acid	0.1	4.1
Threonine	0.1	0.4
Serine	1.0	0.5
Asparagine		—
Glutamine		—
Glutamic acid	0.1	3.5
Proline	0	0
Glycine	0.2	3.7
Alanine	10.0	10.5
Half-cystine	0	0
Valine	3.7	3.6
Methionine	0	0
Isoleucine	0.1	0.4
Leucine	0.2	0.3
Tyrosine	0	0
Phenylalanine	0	0
Tryptophan	0	—
Lysine	1.3	6.6
Histidine	0	0
Ammonia	0.3	6.5
Arginine	0	0

[a] The incubation mixture contained the same compositions as in Table III except modifying enzyme. Cpase A, 4.5 mg, was used instead of the modifying enzyme and the reaction was carried out at 38° for 150 minutes. Amino acid analyses were performed on the 5 % TCA-soluble fraction (see Table III).

[b] Hydrolysis of the 5 % TCA-soluble fraction was carried out in 6 N HCl for 24 hours at 105°.

Products during the modification of native PGA mutase by untreated Cpase A were analysed. As shown in Table VIII, amino acids and peptides released are closely similer to these which were released by the action of modifying enzyme.

This fact suggests that a DFP-sensitive enzyme included in the Cpase A preparation has a modifying enzyme-like activity. Trypsin or chymotrypsin is usually expected as an impurity in the Cpase A preparation. But these endopeptidases did not exhibit the modifying enzyme-like action (Table VII).

An alternative possibility should be taken into consideration ; the modifying enzyme-like activity is revealed by a cooperative action of Cpase A and chymotrypsin or trypsin. To test this possibility, the DFP-treated Cpase A preparation was dialysed against 0.02 M phosphate buffer, pH 7.5. The dialyzate obtained was free from excess DFP. Unfractionated PGA mutase was incubated with trypsin and DFP-treated and dialysed Cpase A mixture, or chymotrypsin and DFP-treated and dialysed Cpase A mixture.

Incubation mixtures contained 1.8 mg/ml PGA mutase, 1.3 mg/ml Cpase A treated as described above and 8.2 μg/ml trypsin or 4.5 μg/ml chymotrypsin. The reactions were carried out in 0.02 M sodium phosphate buffer, pH 7.5 for 30 minutes at 38°. As a result, modification of PGA mutase was not observed. Therefore, it seems unlikely that PGA mutase is modified with a cooperative action of Cpase A and chymotrypsin or trypsin.

Neurath et al. reported that the appearance of the intermediate which had endopeptidase activity and was sensitive to DFP was observed during a trypsin-catalyzed activation of bovine pancreatic Cpase A (30). An enzyme having modifying enzyme-like activity may be an endopeptidase appeared in the course of the activation.

4. Discussion

The term "isozyme" has been proposed for enzymes derived from the same source that have the same catalytic action, but different physical and chemical properties (31). Observations on isozymes have been made by many investigators (32,33). It is possible that enzymic and artificial modification of an enzyme may occur in the course of extraction and purification. These possibilities should be carefully examined before a decision is made as to the existence of multiple forms of an enzyme.

Although crystalline PGA mutase obtained from an autolysate of baker's yeast is composed of multiple forms, several observations showed that native PGA mutase is component I and that this is modified enzymatically during autolysis (13, 17). This conclusion was supported by the fact that crystalline PGA mutase from an acetone powder of yeast by low-temperature extraction consisted almost entirely of component I. The modifying enzyme may not attack PGA mutase in intact cells because of different localization or because it

exists as an inactive precursor.

It is concluded that the modification process is a limited proteolysis and that the differences in electrophoretic mobility of individual components derive from differences in lysine content. As described in Chapter V, the optical rotatory dispersion parameters of native PGA mutase imply that this enzyme has a highly compact structure. Measurements of optical rotation suggested that the conversion of native to modified PGA mutase is not accompanied by major structural changes. The fact that there was no change in the ultraviolet difference spectrum during modification means that there is no alteration in the environment of tyrosine and tryptophan residues. Additionally, all crystalline preparations of PGA mutase have the same crystalline form and show the same 2,3-PGA phosphatase activity. From these results, it is presumed that PGA mutase retains its conformation even after suffering degradation by the modifying enzyme.

Usually, proteins degraded by limited proteolysis have different crystalline forms (34,35) or can not be crystallized under the same conditions as the native proteins (36). It is noteworthy that each component of PGA mutase exhibits the same crystalline form.

CHAPTER IV

CHEMICAL MODIFICATION

1. Introduction

Chemical modification experiment is an excellent technique in the investigation of amino acid residues constructing the active center of an enzyme protein. It is required that denaturation of the enzyme under conditions of modification reactions and conformational change due to masking or distruction of amino acid residues does not occur.

In this chapter, the effects of various chemical modifiers on PGA mutase activity are studied. It is concluded that lysine residues have an significant role on PGA mutase activity.

2. Materials and Methods

Native PGA mutase (component I) was used throughout all experiments described in this Chapter.

3. Results and Discussion

Serine, Threonine and Cysteine

Diisopropylfluorophosphate (DFP) is an organophosphorus compound which reacts specifically with serine and threonine residues in protein (37). Since PGA mutase activity was not affected by the incubation with DFP as shown in Table IX, serine and threonine residues have not direct contribution to formation of the active center of PGA mutase.

Table IX

Chemical Modification of PGA Mutase[a]

Reagent	Sensitive groups	Reagent / PGA mutase (mol.ratio)	Concn. of PGA mutase	Conditions °C, pH, Min.	Inhibition (%)
DFP	Ser, Thr	100	1×10^{-5}M	25, 7.5, 60	0
p-CMB	-SH	10	1×10^{-5}M	25, 7.5, 60	0
		2000	5×10^{-7}M	37, 7.5, 20	0
		2000	5×10^{-7}M	37, 6.0, 20	0
		10^5	5×10^{-8}M	37, 6.0, 20	60

[a] Native PGA mutase (component I) was used.

As desrcibed in Chapter III, one mole of sulfhydryl group exists in one mole of PGA mutase. Incubation of PGA mutase with p-chloromercuribenzoate which is a specific modifier of sulfhydryl group did not change the enzyme activity. Under the condition of the low protein concentration, however, PGA mutase activity was lowered. Since PGA mutase protein was diminished in its stability under the condition, it appeared that the loss of activity was attributable to denaturation during the modification reaction. It is concluded that sulfhydryl group is not essential for the action of PGA mutase.

Lysine

Trinitrobenzenesulfonate (TNBS) is an usefull reagent to investigate the role of primary amino groups in an enzyme protein, for it reacts specifically with amino groups under a mild condition and its derivatives exhibit the absorption spectra at around 345 mμ (25). The amount of TNBS bound to PGA mutase was calculated from absorption at 345 mμ with a molecular extinction

coefficient of 1.33×10^4 (25). The rate of trinitriphenylation in borate buffer, pH 7.5 at 0° was shown in Fig. 8.

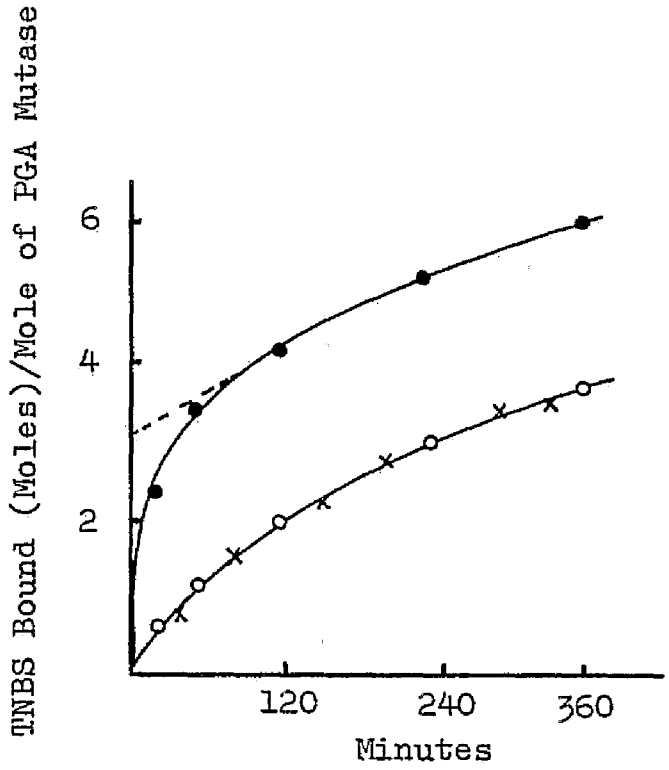


Fig. 8. Trinitrophenylation of PGA Mutase.

The reaction mixtures contained 5×10^{-6}M protein, 5×10^{-4}M TNBS, 0.035 M sodium borate-HCl buffer (pH 7.5) and DL-2-PGA or D-3-PGA at the following concentrations. (o), 10^{-3}M DL-2-PGA ; (x), 10^{-3}M D-3-PGA ; (•), none. The temperature was 0°. Absorption at 345 mμ was measured by using the technique of differential spectrophotometry. Reference cells contained all reagents except PGA mutase. Prior to the reaction, native PGA mutase was subjected to gel filtration on a Sephadex G-50 column.

PGA mutase have three amino groups which react rapidly with TNBS. Since NH_2-terminal amino acid residues could not be detected by using Levy's dinitrophenylation method in which the dinitrophenylation reaction was carried out in aqueous solution at pH 8.0, it appears that terminal amino acid residues are acetylated or buried in the interior of the molecule. Therefore, three amino groups, reactive for TNBS, are attributable to ε-amino groups of lysine residues. The trinitrophenylation reaction was strongly prevented by DL-2-PGA or D-3-PGA (Fig. 8). The data presented in Fig. 8 indicate that substrates of PGA mutase protect two lysine residues. As shown in Fig. 9, the trinitrophenylation caused inactivation of PGA mutase. On the other hand, DL-2-PGA or D-3-PGA preserved completely enzyme activity. Thus, it is concluded that two of three lysine residues trinitrophenylated rapidly are located in the active site region.

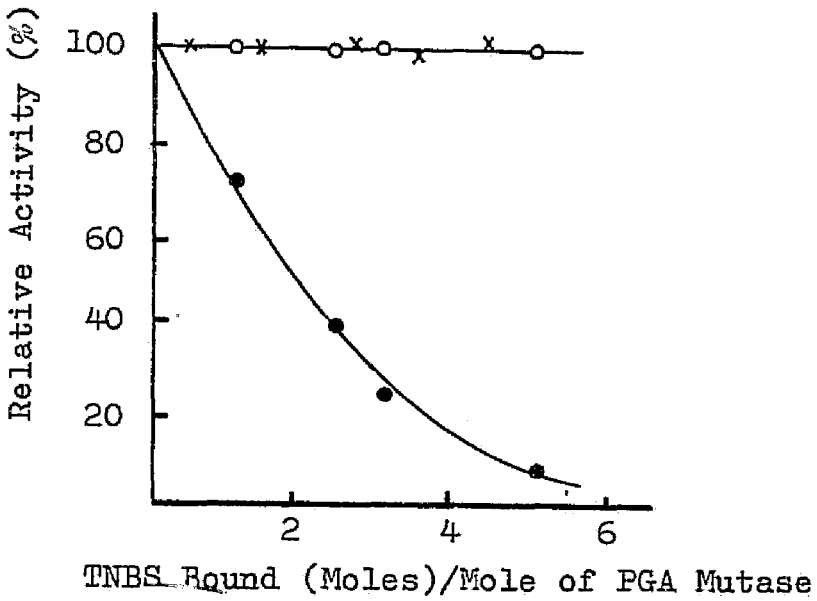


Fig. 9. The Effect of Substrates on Inactivation of PGA Mutase by TNBS.

The reaction mixtures and conditions were the same as in Fig. 8. The reaction mixtures contained DL-2-PGA or D-3-PGA at the following concentrations.
(o), 10^{-3}M DL-2-PGA ; (X), 10^{-3}M D-3-PGA (•), none. Aliquots were diluted in cold 0.02 M ammonium sulfate solution at intervals and PGA mutase activity was determined.

CHAPTER V

CONFORMATIONAL STUDY

1. Introduction

Many experimental methods have been developed to help in the elucidation of the structure of the protein molecule in aqueous solution. However, no single technique or set of conditions will provide the answers to the questions. Since it is our ultimate aim to understand the process which occur in biological systems, we tend to focus our attention on reaction in which conditions (solvent, pH, ionic strength, etc) are close to these obtaining in the living system. However, in order to clarify relation between structure and catalytic activity of enzymes, we must not restrict ourselves to these conditions. It is possible to presume native protein structures through experiments carried out at extremely acid or alkali pH, in denaturing solvents, etc.

In this chapter, the data obtained by otical rotatory dispersion and ultraviolet

difference spectrum measurements and ultracentrifugal analyses of PGA mutase are described. Urea denaturation and its reversibility are also studied.

2. Materials and Methods

Native PGA mutase (component I) was used throughout all experiments described in this Chapter. Optical rotatory dispersion measurements in the range of 320—600 mμ were obtained with a photoelectric polarimeter attached to a Beckman DU spectrophotometer, and with a Rudolph photoelectric spectropolarimeter. A Beckman DU spectrophotometer was used to measure ultraviolet difference spectra. Sedimentation analyses were performed with a Spinco analytical ultracentrifuge.

3. Results

a. Optical Rotatory Dispersion

Optical rotation of polypeptides and proteins is based on the peptide bond, for it is the spatial disposition of this bond that gives rise

to the rotatory characteristics of these polymers and defines their secondary structure. Optical rotation measurement is an excellent technique to clarify protein structures because of the special sensitivity of optical rotatory power to mutual orientation of peptide groups

It is often used to measure rotatory power at one wavelength, but monochromatic data do not offer satisfactory informations about the conformation of protein molecule. Optical rotatory dispersion, that is, the variation of optical rotatory power with wavelength, provide much advantageous knowledge that may be correlated with structure.

In early studies, interpretation on the dispersive properties of native proteins had been based on the hypothesis by which native proteins were consisted of helical and disordered regions. It was supported cleary from X-ray diffraction studies of myoglobin by Kendrew et al (38). Recently, several cases have been found in which it appears unable to explain the observations by this simple hypothesis.

Two hydrogen-bonded structures, helices of opposite sense and β-structure, are provided for new explanation of these observations.

Optical rotatory dispersions of native and denatured PGA mutase, however, can be explained by the hypothesis that the native structure of PGA mutase in aquous solution is primarily the result of hydrophobic forces uniquly associated with water as a solvent.

Figure 10 shows optical rotatory dispersions of PGA mutase in phosphate buffer, pH 7.0 and 8 M urea solution, pH 7.0. A more positive rotation ($(\alpha)_D$ = -16°) for native protein suggests that this protein is in highly folded state in aqueous solution.

The observed dispersions of many proteins in the visible spectrum obey the simple Drude equation.

$$[\alpha]_\lambda = \frac{K}{\lambda^2 - \lambda_c^2} \qquad [\alpha]_\lambda \lambda^2 = [\alpha]_\lambda \lambda_c^2 + K$$

When $[\alpha]_\lambda \lambda^2$ is plotted against $[\alpha]_\lambda$, the slope of a straight line obtained gives λ_c^2 .

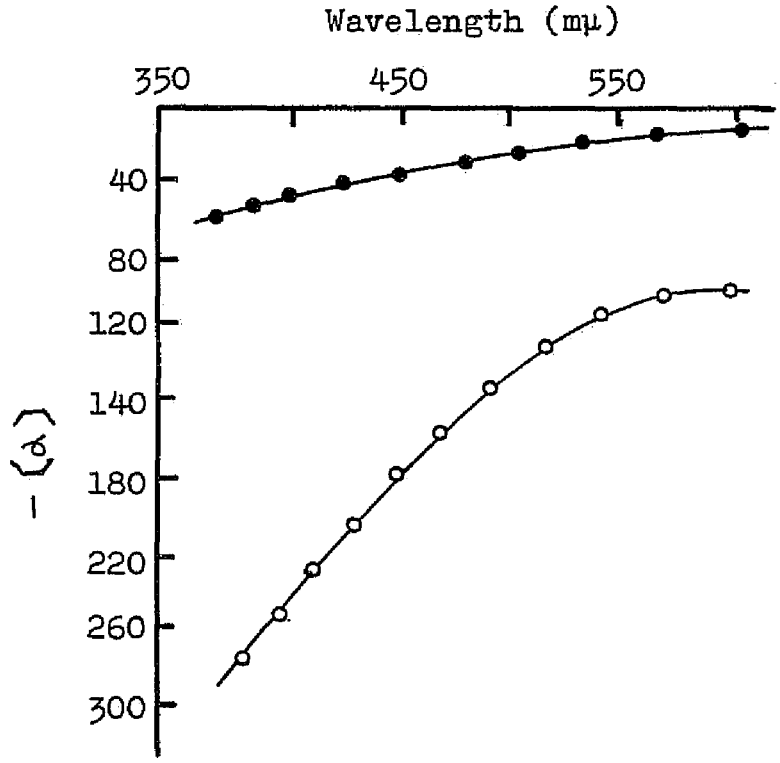


Fig. 10. Optical Rotatory Dispersion of PGA Mutase.

(•) Values determined in 0.01 M phosphate buffer, pH 7.0 ; (○) values determined in 8 M urea at pH 7.0 containing 0.01 M phosphate buffer.

The quantity λ_c can be used to estimate percent helical content, but only up to about 50 % helical content. Based on data for poly-L-glutamic acid by Yang and Doty (39), the lower limit for λ_c ,

corresponding to the completely random coil, is about 212 mμ ; the value of λ_c increases to a value of 285 mμ for 50 % helix. The results plotted according to Drude equation are shown in Fig. 11. Based on data of Yang and Doty (39), the value of λ_c (=242 mμ) obtained for native protein shows that helical region of PGA mutase is about 20 %.

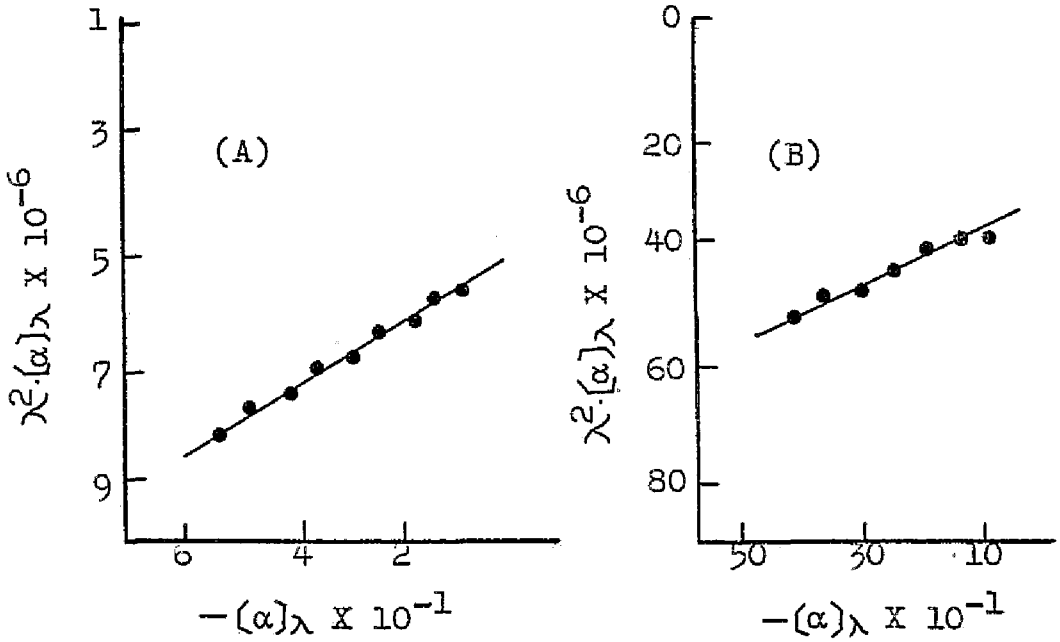


Fig. 11. Optical Rotatory Dispersion of PGA Mutase Plotted According to the Drude Equation.

(A) Values determined in 0.01 M phosphate buffer, pH 7.0 ; (B) values determined in 8 M urea at pH 7.0 containing 0.01 M phosphate buffer.

Moffit and Yang (40) developed a phenomenological equation to treat the empirical data of helical polymers and derived an expression that is analogous to the simple Drude equation. Moffit's resulting equation is

$$[m']_\lambda = \frac{\bar{M}}{100}\frac{3}{n^2+2}[\alpha]_\lambda = \frac{a_o\lambda_o^2}{\lambda^2-\lambda_o^2} + \frac{b_o\lambda_o^4}{(\lambda^2-\lambda_o^2)^2}$$

where $[\alpha]_\lambda$ is the specific rotation at wavelength λ, $[m']_\lambda$ is the mean residue rotation at λ, $\bar{M}$ is residue weight, n is the refractive index of the solvent, and a_o, b_o and λ_o are composite constants pertaining to the optically active absorption bands. The constant b_o is characteristic of the helix, and the a_o term contains both helix and residue contributions. The λ_o determined experimentally by Moffit and Yang is 215 $\pm$ 5 mμ (40). If $\lambda^2 - \lambda_o^2/\lambda_o^2 \cdot [m']_\lambda$ is plotted against $\lambda_o^2/\lambda^2 - \lambda_o^2$, the second term constant b_o in this equation is obtined from the slope of an straight line and the first constant is obtained from the intercept on $\lambda^2 - \lambda_o^2/\lambda_o^2\ [m']_\lambda$ axis.

On the assuption that the b_o value for a completely helical protein would be -630, the percent 'helix is calculated as follows ; the percentage of helical content = $b^{obs.}$/ -630 X 100.

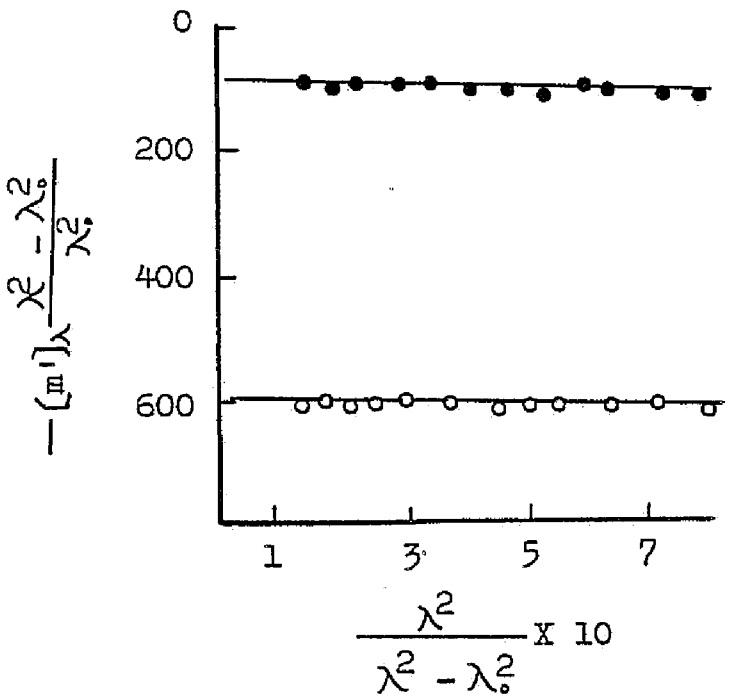


Fig. 12. Optical Rotatory Dispersion of PGA Mutase Plotted according to the Moffit-Yang Equation.

(•) Values determined in 0.01 M phosphate buffer, pH 7.0 ; (o) values determined in 8 M urea at pH 7.0 containing 0.01 M phosphate buffer.

The data of PGA mutase in terms of Moffit-Yang equation are shown in Fig. 12. Here, n, the refractive index of the solvent, is 1.334 for 0.01 M phosphate buffer, pH 7.0 and 1.46 for 8 M urea ; $\bar{M}$ is taken here as 115 ; and λ_o is assumed equal to 212 mμ. The resulting values of a_o and b_o are listed in Table X.

Table X
Optical Rotatory Dispersion Parameters

		λ_c (mμ)	b_o	a_o	$[\alpha]_D$
PGA mutase	Native	242	-38	-95	-16°
	Urea denatured	212	-17	-604	-104°
β-Lactoglobulin[a]	Native	249	-68	-169	-28°
	Urea denatured	225	-51	-663	-117°

[a] These values are from Tanford et al (41).

As shown in Table X, the optical rotatory dispersion behavior of native and urea-denatured PGA mutase resembles that of β-lactoglobulin (41). The value of b_o, a parameter for α-helical content, is -38 for native PGA mutase.

If a value for b_o of -630 is accepted for the completely helical state and a value of 0 for the absence of helix, PGA mutase may be referred to as "nonhelical".

b. States of Tyrosyl and Tryptophyl Residues

Solvent Perturbation Spectra

It is likely that binding sites of substrate, conenzyme or cofactor are located on the surface of the protein. Therefore, it is important to estimate the number of amino acid residues which are exposed and accessible to the solvent in the native protein molecule. The absorption of proteins in ultraviolet region (260 ~ 300 mμ) has been attributed to phenylalanyl, tyrosyl and tryptophyl residues. Small changes in the solvent composition of aqueous solution of these chromophores generally cause small shifts in the absorption spectra of chromophores. Herskovits and Laskowski (42,43) reported that exposed and buried chromophoric groups in proteins are distinguishable by using the solvent perturbation spectrum technique.

The spectra of groups exposed to the exterior of the protein molecule should be affected by solvent perturbations and these of buried groups should not be affected, provided the conformation of the protein remains unaffected by the additive. The small spectral shifts due to solvent perturbations can be measured accurately by the technique of differential spectrophotometry.

The solvent perturbation technique of Herskovits and Laskowski was applied to estimate the number of tyrosyl and tryptophyl residues in PGA mutase which are accessible to the solvent. The difference spectrum between two protein solutions, one in an aqueous environment and the other in the presence of a perturbant was measured. The difference spectra of N-acetyl-DL-tryptophan and N-chloroacetyl-L-tyrosine produced by 20 % polyethylene glycol are shown in Fig. 13 and that of PGA mutase in Fig. 14. These results are summarized in Table XI.

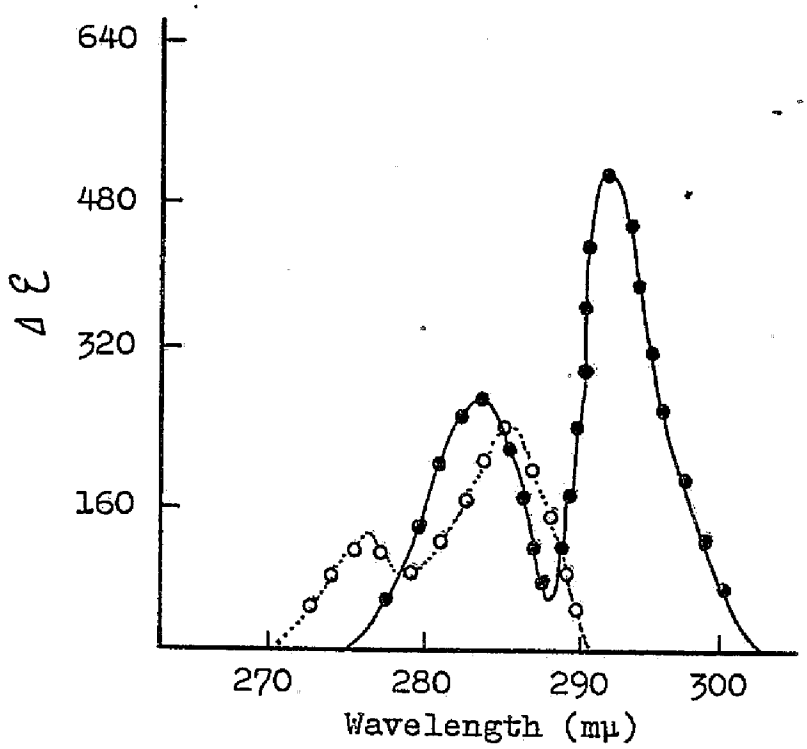


Fig. 13. Solvent Perturbation Difference Spectra of Model Compounds due to 20 % Polyethylene Glycol.

Solid curve, N-acetyl-DL-tryptophan ; dotted curve, N-chloroacetyl-L-tyrosine. These spectra were determined at a concentration of 2.9×10^{-4}M for tryptophan derivative, 1×10^{-3}M for tyrosine derivative, in neutral solution.

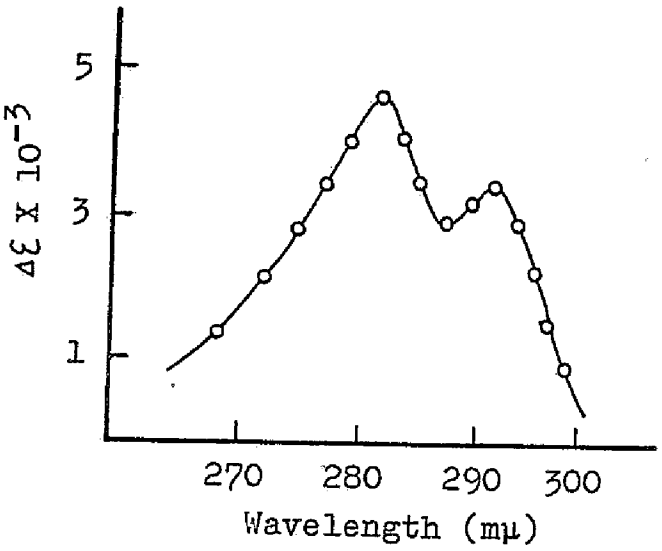


Fig. 14. Solvent Perturbation Difference Spectrum of PGA Mutase due to 20 % Polyethylene Glycol.

The spectrum was determined at a concentration of 1.03 mg/ml PGA mutase in 0.01 M phosphate buffer, pH 7.0.

Table XI

Summary of Difference Spectral Results

Compound	Δε at	
	285 mμ	292 mμ
Tryptophan derivative	246	505
Tyrosine derivative	204	nearly zero
PGA mutase	4341	3144

In order to estimate the amounts of chromophoric residues in PGA mutase which are accessible to polyethylene glycol, simultaneous equations were solved.

$$246Y + 204X = 4341 \text{ ----- (1)}$$

$$505Y = 3144 \text{ ----- (2)}$$

$$Y = 6.2, \quad X = 13.7$$

In these equations, X represents moles of tyrosyl residues which are exposed in one mole of PGA mutase and Y represents those of tryptophyl residues. As PGA mutase activity was not influenced by the presence of 20 % polyethylene glycol, it is probable that the enzyme molecule is not denatured in the presence of this perturbant. Of the 35 tyrosyl groups in native PGA mutase, fourteen are exposed and accessible to the perturbant. In the case of tryptophyl groups, about 6 of the 16 tryptophyl groups are exposed.

Urea Difference Spectra

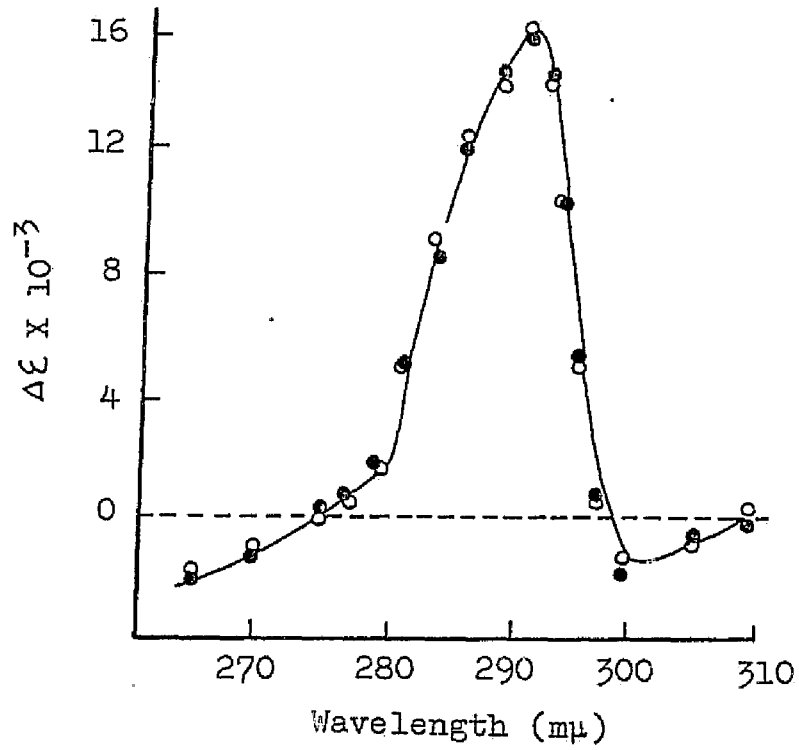


Fig. 15. Urea Difference Spectrum. The sample solution was native enzyme in 0.01 M phosphate buffer, pH 7.0. The reference solution was enzyme in 8 M urea solution of pH 7.0 containing 0.01 M phosphate buffer. Concentration of enzyme was 2.5 mg/ml. (•) Values obtained after 1 hour in 8 M urea ; (o) values determined after 24 hours.

A blue shift in the ultraviolet spectrum of PGA mutase appears in 8 M urea, and enzymic activity is completely lost. Figure 15 shows difference spectra between native and denatured PGA mutase.

These spectra show that tyrosyl and tryptophyl residues buried in the interior of the molecule are brought into contact with solvent by unfolding the native enzyme. The difference spectrum did not change on prolongation of exposure to 8 M urea.

c. Sedimentation Analysis

Sedimentation analysis of PGA mutase in 8 M urea solution at pH 7.0 was carried out in a synthetic boundary cell. A single symmetrical boundary was observed even after 5 hours at 59,780 rpm. The value of $s_{20,\,w}$ (1 % solution) is 1.3S, which suggests dissociation into subunits. As described in Chapter III, the value of $s_{20,\,w}$ (0.7 % solution) in native state is 5.7S.

No change in the sedimentation constant was observed in 1 M urea. As shown in Fig. 16, in 2 and 3 M urea, partial dissociation was observed. The enzymic activity changed in proportion to the content of native enzyme (Table XII, Fig. 16). In 4 M or higher concentration of urea, PGA mutase appeared to be completely dissociated and inactivated.

At 2 or 3 M urea, the dissociation and inactivation were time-dependent (Fig. 16, Table XII).

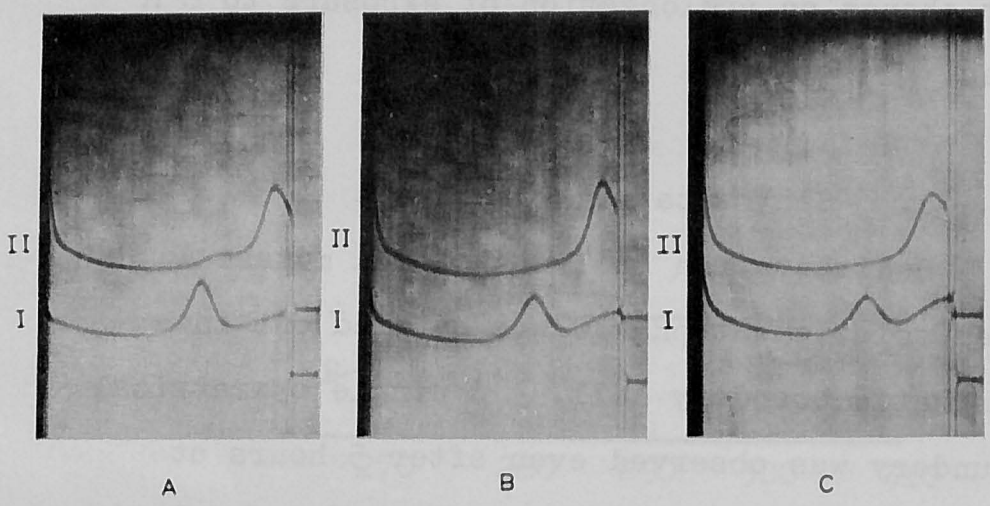


Fig. 16. Sedimentation Velocity Patterns for PGA Mutase Solutions in the Presence of Several Concentrations Urea.

All solutions contained PGA mutase at a concentration of 6.16 mg/ml. All pictures were taken 60 minutes after reaching a speed of 59,780 rpm, with the angle of the shlieren diaphragm at 70°. After preparation the solutions were kept for 5 hours (A,B) and 7 days (C) before analysis in the ultracentrifuge. A-I, PGA mutase in 0.1 M phosphate buffer containing 1 M urea, pH 7.0. A-II and C-II, PGA mutase in 0.1 M phosphate buffer containing 3 M urea, pH 7.0. B-I and C-I, PGA mutase in 0.1 M phosphate buffer containing 2 M urea, pH 7.0. B-II, PGA mutase in 0.1 M phosphate buffer containing 4 M urea, pH 7.0.

Table XII

Enzyme Activity of PGA Mutase Exposed to Urea[a]

Urea concn. (M)	Relative enzyme activity		
	2-hours exposure	6-hours exposure	7 days exposure
0	100	100	85
1	91	88	75
2	71	67	44
3	43	20	0
4	0	0	0
5	0	0	0
8	0	0	0

[a] Urea was dissolved in 0.1 M phosphate buffer, pH 7.0.

d. Reversibility of Urea Denaturation

Anfinsen White have suggested from studies on ribonuclease that the ordered conformation of proteins is directed by the primary structure (44,45). This suggests that reversible denaturation of enzymes would be expected under suitable condition of refolding. Some exsamples of reversible denaturation are known in which disulfide bonds are reformed (44-48). Renaturation of enzymes which have no disulfide bonds has also been reported (49-54).

As described already, rapid unfolding of PGA mutase in 8 M urea was observed by optical rotation and ultraviolet absorption measurements. Urea-denatured PGA mutase, however, is reactivated by dilution or dialysis under suitable conditions.

Reactivation of Urea-denatured PGA Mutase by Dilution or Dialysis

Urea-denatured PGA mutase which had been completely inactivated promptly recovered up to 85 % of its original activity on dilution with 0.2 M sodium phosphate buffer, pH 7.0.

Similer reactivation occurred on dialysis against the same buffer. However, when the enzyme concentration was higher than 0.1 %, precipitation occurred. It is suggested that irreversible aggregation was caused by an intermolecular reaction at higher concentrations of the denatured enzyme.

Effect of Various Diluents on Reactivation

After PGA mutase was kept in 8 M urea solution for one hour at 20°, the solution was diluted in various ways and avtivity determined after 30 minutes. As shown in Table XIII, more than 40 % of the original activity was regenerated in these treatments. Phosphate buffer at pH 7.0 was most effective. Unless otherwise indicated, it was used as diluent in the following experiments.

Table XIII

Effect of Various Diluents on Reactivation[a]

Diluent	pH	Ionic strength	Recovery of activity (%)
phosphate buffer	7.0	0.44	85
phosphate buffer	7.0	0.22	80
phosphate buffer	7.0	0.04	58
NaCl	7.0	0.33	50
NH_4Cl	7.0	0.33	54
$(NH_4)_2SO_4$	7.0	0.30	71
Na_2SO_4	7.0	0.30	73
veronal buffer	7.0	0.05	41
tris buffer	7.5	0.04	59

[a] A 0.1 % PGA mutase solution in 8 M urea was kept at pH 7.0 for 60 minutes at 20°. Then the solution was diluted as indicated to 0.005 % enzyme concentration. Enzyme activities were determined after 30 minutes standing at 20°.

Effect of Concentration and pH Diluent on Reactivation

Reactivation depended on phosphate concentration and occurred rapidly. Although the effect of enzyme concentration in 8 M urea was also examined, it was negligible.

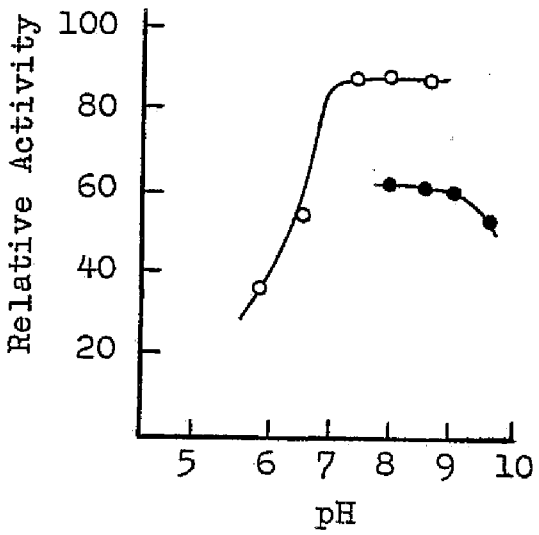


Fig. 17. Effect of pH of Diluent on Reactivation.

Conditions of exposure to 8 M urea were the same as those indicated in Table XIII. Conditions of dilution and activity determination were also the same as those indicated in Table XIII, except that buffers with various pH values were used as diluent. (•) 0.05 M tris buffer ; (o) 0.2 M phosphate buffer.

Reactivation was also examined in 0.2 M phosphate and 0.05 M tris buffers at different pH values. The conditions of exposure to 8 M urea and dilution were the same as described in Table XIII. As shown in Fig. 17, maximum reactivation occurred in the pH region between 7.0 and 8.5.

Effect of Exposure Period in
8 M Urea on Reactivation

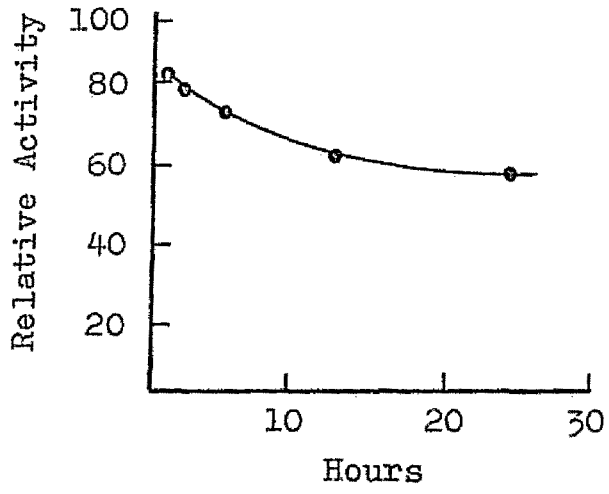


Fig. 18. Effect of Standing in 8 M Urea on Reactivation.

A 0.1 % PGA mutase solution in 8 M urea, pH 7.0, was sampled at various times at 20°. Conditions of dilution and activity determination were the same as those indicated in Table XIII except that 0.2 M phosphate buffer, pH 7.0, was used as diluent.

The extent of reactivation decreased on prolongation of exposure to 8 M urea (Fig. 18). After 24 hours, 55 % of the original activity was recovered as opposed to 85 % after 1 hour.

This result suggests that further conformational alteration occurs on prolongation of the exposure. As described before (Fig. 15), however, the difference spectrum in 8 M urea did not change with time, nor did the optical rotatory behavior.

Preparation and Properties of Regenerated Crystalline PGA Mutase

A 0.4 % native PGA mutase solution in 8 M urea was kept for 30 minutes at 20°, then diluted twentyfold with 0.2 M sodium phosphate buffer of pH 7.0. After 30 minutes the solution was brought to 0.75 saturation with ammonium sulfate. The suspension was centrifuged at 0° for 30 minutes at 10,000g and the precipitate was collected. The precipitate was dissolved in an adequate volume of 0.1 M sodium phosphate buffer at pH 7.0. The insoluble portion corresponded to about 15 % of the total protein. The percentage solubilized (85 %) was equivalent to the regenerated activity. Moreover, the specific activity of the soluble fraction was equal to that of the native enzyme.

The regenerated enzyme was crystallized by the method of Chiba and Sugimoto (18). The crystalline form was the same as that of the native enzyme.

Table XIIII

Properties of Native and Regenerated PGA Mutase

Property	Native PGA mutase	Regenerated PGA mutase
Relative activity	100	98
Ultraviolet spectrum	Identical	
Optical rotatory dispersion parameters		
λ_c	242 mμ	245 mμ
a_o	-95	-75
b_o	-38	-50
$(\alpha)_D$	-16°	-15°
$s_{20,w}$ (1.1 % solution)	5.6	5.5
Keq (3-PGA/2-PGA)	5.2	5.3
pH optimum	5.9	5.9
Crystalline form	rhombic plate	rhombic plate

Physicochemical and enzymic properties of the native and regeberated PGA mutase were compared.

As indicated in Table XIIII, both preparations showed virtually identical specific activities, ultraviolet absorption spectra, optical rotatory dispersion parameters (λ_c, a_o, b_o, and $[\alpha]_D$), sedimentation constants, pH-activity curves, and equilibrium constants for the reaction catalyzed (3-PGA/2-PGA).

4. Discussion

Although native PGA mutase in phosphate buffer at pH 7.0 posseses a more positive rotation ($(\alpha)_D$ = -16°) than most globular proteins ($(\alpha)_D$ = -20° ~ -60°), the value of b_o obtained from optical rotatory dispersion was unexpectedly low, only -38. On denaturation in urea, the value of a_o changed from -95 to -604, but the value of b_o did not alter appreciably. If regular structures are assumed to be absent in native PGA mutase, the remarkable change of the value of $(\alpha)_D$ or a_o upon denaturation may be explained by the presence of hydrophobic regions as in β-lactoglobulin (41). The more positive value of $(\alpha)_D$ in the native enzyme suggests that the majority of peptide bonds are buried in the hydrophobic inner portions inaccessible to a solvent. If this concept is correct, the enzyme would appear to be nonhelical. It is also possible that in the native conformation PGA mutase contains right- and left-handed α-helices in equal proportions, or posseses both α-helices and β-structures.

Various data indicate that most polypeptides derived from L-amino acids can exist predominantly in the form of right-handed α-helices under some experimental conditions. An exception is poly-β-benzyl-L-aspartate which exists as a left-handed α-helix (55,56). The existence of a left-handed α-helix stabilized by disulfide bonds has been proposed in insulin (57,58). The value of a_o and b_o of the Moffit equation for β-conformations have been estimated by several investigators (59,60) since Yang and Doty (61) indicated that the value of these parameters are positive. If α-helix and β-structures are present in about equal amounts, b_o must be small. An expression for the optical rotation of a mixture of helices, β-form, and disordered regions can be derived in the same way as Moffit-Yang equation.

$$[m']_\lambda = \frac{(a_o^R + f_H a_o^H + f_\beta a_o^\beta)\lambda_o^2}{\lambda^2 - \lambda_o^2} + \frac{(f_H b_o^H + f_\beta b_o^\beta)\lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

If $\lambda^2 - \lambda_o^2/\lambda_o^2 \cdot [m']_\lambda$ is plotted against $\lambda_o^2/\lambda^2 - \lambda_o^2$, the first term, $a_o^R + f_H a_o^H + f_\beta a_o^\beta$, is obtained from the intercept on $\lambda^2 - \lambda_o^2/\lambda_o^2 \cdot [m']_\lambda$ axis and the second term, $f_H b_o^H + f_\beta b_o^\beta$, from the slope of an straight line.

Here, the value of a_o^R is obtained from the intercept on $\lambda^2 - \lambda_o^2/\lambda_o^2 \cdot [m']\lambda$ axis when the data in a completely denatured protein are plotted according to Moffit - Yang equation ; f_H and f_β are fractions of helix and β-form respectively ; a_o^H and b_o^H are characteristic constants for helix ; a_o^β and b_o^β are characteristic constants for β-form. If I use the standard helical constants (a_o^H = + 700, b_o^H = -600), the Wada constants for β-form (a_o^β = + 840, b_o^β = + 420)(59), and the parameters shown in Table X (a_o^R = -604, $a_o^R + f_H a_o^H + f_\beta a_o^\beta$ = -95, $f_H b_o^H + f_\beta b_o^\beta$ = -38), simultaneous equations described below are introduced.

$$700f_H + 840f_\beta = 509 \text{ ----- (1)}$$

$$-600f_H + 420f_\beta = -38 \text{ ----- (2)}$$

Therefore, f_H is 0.31 and f_β is 0.35. However, because of discrepancies in the reported values of the parameters for the β-form, it is impossible to evaluate the content of β-conformations reliably.

PGA mutase appears to be quite sensitive to urea, for even 2 M urea causes partial denaturation and dissociation.

At higher urea concentration (> 4 M), denaturation and dissociation of the enzyme occur rapidly. It is evident that noncovalent interactions exist between several subunits. Since the sedimentation pattern in 8 M urea showed a single boundary, subunits may be similer molecular weights. The presence of only one cysteine residues, however, suggests that the subunits are not identical.

After the enzyme was denatured and dissociated in 8 M urea, a remarkable reconstitution occurred on dilution or dialysis. The nature and concentration of ions, and the pH affected the extent of reconstitution. A high concentration of phosphate was effective, though the role of phosphate has not been clarified. It is possible that high concentrations of ions may be important for screening charges during refolding. The extent of reactivation decreased on prolongation of the exposure to 8 M urea (Fig. 18), possibly because of carbamylation by cyanate formed in concentrated urea solution (62).

The physicochemical and enzymic properties as well as the crystalline form of the regenerated enzyme were identical to those of the native enzyme (Table XIIII). The λ_c value was 212 mμ in 8 M urea. This value is that of a protein in the completely random conformation. Therefore, it appears that the native conformation is reconstituted from the completely random structure and that the specific reconstitution process is not initiated in small ordered regions of the peptide chain. Consequently, it is suggested that the primary structure of the enzyme directs folding to a specific ordered structure and that a "folding template" in the biosynthesis of yeast PGA mutase is not required.

SUMMARY

Relationship between structure and catalytic activity of crystalline yeast PGA mutase were investigated. These results are summarized as follows.

In Chapter III, mechanism of enzymic modification of PGA mutase is clarified. Crystalline PGA mutase preparations obtained from autolyaste of baker's yeast contain five components which are electrophoretically distinguishable and which exhibit different specifc activities. These components are not isozymes. One of these components is the native protein (component I) and the others arise from the action of PGA mutase -modifying enzyme during autolysis. The enzyme which modifies PGA mutase to produce components (II, III, IV, and V) with higher electrophoretic mobilities and lower specific activities than native enzyme has been shown to be a peptide hydrolase. Quantitative measurements have indicated that amino acids (lysine, asparagine, glutamine, alanine, valine, and glycine) are

liberated during the conversion of component I to the limit product (component V). It has been shown that electrophoretic mobilities of the modified components correlate with the liberation of lysine. Major conformational changes have not been observed during the modification. Some properties of the modifying enzyme, and of component I and V of PGA mutase are presented.

In Chapter IV, amino acid residues constructing the active center of PGA mutase are studied by chemical modification experiments. Two lysine residues have a significant role in the action of PGA mutase. Serine, threonine, and cysteine residues are not essential for the action of PGA mutase.

In Chapter V, three dimensional structure of PGA mutase are studied by using several techniques developed recently. The structure of PGA mutase in phosphate buffer and in urea solutions has been investigated by ultraviolet spectra, optical rotatory dispersion measurements, and ultracentrifugal analyses. The results suggest that the native enzyme may be "nonhelical"

and that the conformation is stabilized by hydrophobic forces associated with water as a solvent. Other possibilities have also been discussed. The native enzyme, which has no disulfide linkages, is unfolded and dissociates into subunits at concentration greater than 2 M urea. Reactivation and reconstitution of the enzyme denatured in 8 M urea by dilution or removal of urea have been observed. Regeneration depends on the pH and on the nature and concentrations of ions in the diluents. Comparison of the physicochemical and enzymic properties has suggested that the reconstituted and native enzymes are identical.

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